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(54) **PDE core construct**

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Construction dérivée du noyau de PDE

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Description

[0001] The invention relates to modified phosphodiesterase (PDE) polypeptides with altered physicochemical properties which are particularly useful in screening assays for the identification of agonists or antagonists of PDEs, the polynucleotides encoding such modified PDE polypeptides, antibodies against such polypeptides, methods of preparation and uses of such polypeptides.

[0002] The phosphodiesterases (PDEs) represent a family of enzymes that catalyze the hydrolysis of the various cyclic nucleoside monophosphates (including cAMP). These cyclic nucleotides have been found to act as second messengers within the cells by carrying impulses from cell surface receptors having bound various hormones and neurotransmitters to the inside of the cells. The task of phosphodiesterases is to degrade these cyclic mononucleotides once their messenger role is completed thereby regulating the level of cyclic nucleotides within the cells and maintaining cyclic nucleotide homeostasis.

[0003] Ten families of PDEs have been identified, designated PDE1 to PDE10. Within each family there are two or more related but distinct gene products (A, B, C, etc.) and for each of these alternative mRNA processing gives rise to multiple splice variants, identified by an additional arabic numeral in accordance with the most recent nomenclature recommendation (Molecular Pharmacology 46, 399-405, 1994).

[0004] All PDE gene products identified so far have one catalytic and a least one regulatory domain. The catalytic domain lies towards the carboxylic acid terminus of each PDE protein and has the greatest homology between the PDE families, being >75% homologous at the amino acid level (Perry and Higgs, Curr. Opin. Chem. Biol. 2, 472-481, 1998). Nevertheless, each of the more than 30 known PDEs have individually distinct substrate specificities, kinetic characteristics, regulatory properties and cellular and subcellular distributions (Houslay and Milligan, Trends in Biochem. Sci. 22, 217-224, 1997).

[0005] PDEs 4, 7 and 8 are highly specific for cAMP. PDEs 5, 6, 9 and 10 are selective for cGMP. PDE3s bind cAMP and cGMP with similar affinity, but hydrolyse cAMP most efficiently, cGMP rather poorly. PDE3s are, therefore, negatively regulated in their cAMP hydrolysing ability by cGMP. PDEs 1 and 2 hydrolyse both cAMP and cGMP, but with PDE1 the relative efficiencies vary with isoenzyme subtype (Perry and Higgs, Curr. Opin. Chem. Biol. 2, 472-481, 1998).

[0006] The amino terminal ends of PDEs consist of the regulatory domains, which are very different both between families and between variants within families. This region contains variously: a binding domain for Ca²⁺-calmodulin (CaM) in PDE1; non-catalytic cGMP-35 binding sites in PDEs 2, 5 and 6; a binding domain for the signalling G-protein transducin in PDE6. The amino terminal region also contains protein- and membrane-targeting sequences in several PDE3s and PDE4s, as well as protein kinase phosphorylation sites in PDEs 1, 3, 4 and 5. These phosphorylation sites are likely to be important in regulation of catalytic activity and/or subcellular location (Perry and Higgs, Curr. Opin. Chem. Biol. 2, 472-481, 1998).

[0007] Of particular interest to this invention are the PDE4 enzymes. PDE4 enzymes share a common structure, as deduced from their amino acid sequences (Beavo and Reifsnnyder, Trends Pharmacol. Sci. 11, 150-155, 1990; Bolger et al., Mol. Cell Biol. 13, 6558-6571, 1993; Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Members of each gene family (PDE4A, PDE4B, PDE4C, PDE4D) share common C-terminal regions, different for each family, and catalytic domains that for all PDE4 isoforms are very similar (84% homology over about 360 amino acids across all PDE4s; Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998).

[0008] From N-terminus to catalytic region, the sequence in "long form" PDE4s can be divided into 5 regions, three of which are isoform-specific (N-terminal region, linker regions 1 and 2, or LR1 and LR2) and two, more conserved regions, that are broadly similar between all isoforms, the upstream conserved regions 1 and 2 (UCR1 and UCR2). "Short form" PDE4s, e.g. PDE4A1, PDE4B2, PDE4D1, PDE4D2, lack UCR1 and LR1 plus differing amounts of the N-terminal region of UCR2. Throughout all regions are potential phosphorylation sites for a variety of kinases, including PKA (e.g. Ser 54 in human PDE4D3), mitogen activated protein kinases (e.g. Ser 487 of human PDE4B2), casein kinase II (e.g. Ser 489 of PDE4B2) and calcium-diacylglycerol dependent protein kinases (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Phosphorylations at some of these sites have been shown to activate the PDEs (e.g. Ser 54), others serve to inhibit. There is also evidence that some phosphorylations serve to prime the enzymes ready for subsequent activation by further phosphorylation at a different site or sites (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Other auto-regulatory sites may be found in the N-terminal sequence of certain PDE4s (Bolger et al., Mol. Cell Biol. 13, 6558-6571, 1993; Mc Phee et al., Biochem. J. 310, 965-974, 1995).

[0009] Specific PDE4 inhibition may be useful over a very wide range of disease areas. These include: asthma, atopic dermatitis, depression, reperfusion injury, septic shock, toxic shock, autoimmune diabetes, AIDS, Crohn's disease, multiple sclerosis, cerebral ischemia, psoriasis, allograft rejection, restenosis, stroke, ulcerative colitis, cachexia, cerebral malaria, allergic rhinoconjunctivitis, osteoarthritis, rheumatoid arthritis, autoimmune encephalomyelitis (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998, WO02/074992). In the area of asthma, PDE4 inhibition helps to increase cAMP in bronchial smooth muscle, thereby producing a modest bronchodilatory effect, of use in the alleviation of asthmatic symptoms. But perhaps most importantly, inhibition of PDE4s is now a recognised method by which to

suppress immune and inflammatory cell responses (Hughes et al. Drug Discov. Today 2, 89-101, 1997; Torphy, Am. J. Respir. Crit. Care Med. 157, 351-370, 1998; Teixeira et al., Trends Pharm. Sci. 18, 164-171, 1997).

[0010] PDE4s play major roles in modulating the activity of virtually every cell type involved in the inflammatory process. Immune and inflammatory conditions occur when recruitment of leukocytes from the blood compartment into tissues is either uncontrolled, inappropriate, prolonged or directed against self. In asthma, rheumatoid arthritis and multiple sclerosis, infiltration of tissues with inflammatory cells is prolonged and intense, leading ultimately to severe (and self-perpetuating) damage and loss of function. Acute dysregulation of the immune system occurs in such conditions as acute respiratory distress syndrome (ARDS) where an overwhelming and generalised inflammatory response can frequently lead to death. There is also substantial evidence which suggests that inflammation may play a part in defining the extent of injury resulting from reperfusion following ischaemia, at least in brain and lung (Entman and Smith, Cardiovasc. Res. 28, 1301-1311, 1994).

[0011] The PDE4D phosphodiesterases, which are the most preferred focus of the present invention, exist in mammals in the form of isoenzymes (which represent different molecular forms of the same enzyme polypeptide). These phosphodiesterases are localized in the cytosol of the cell and are unassociated with any known membraneous structures. The PDE4D isoenzymes specifically degrade cAMP and are a common target for such pharmacological agents as antidepressants (for example, rolipram). Several splice forms of PDE4D are known. The short forms, PDE4D1 and PDE4D2, lack UCR1 and LR1 sites. Rather than these, interest of the present invention focuses on the long isoforms, of which 6 are known, namely PDE4D3, PDE4D4, PDE4D5, PDE4D6, PDE4D7 and PDE4D8. All of these have in common the LR1 and UCR1 sites and the domains located at the C-terminus of these sites, but they have different N-terminal domains. Isoforms PDE4D6, D7 and D8 were recently disclosed in WO02/074992.

[0012] For identification of compounds that inhibit PDE4D isoenzymes, screening assays are used that are either whole cell assays in which the cells are treated with the inhibitor and cAMP hydrolysis is measured, or in vitro assays which use purified PDE4D isoenzymes either isolated from their natural sources or from cells overexpressing recombinant PDE4D isoenzymes. Such assays are described e.g. in WO00/09504, WO01/35979, WO01/68600 and WO98/45268. WO01/35979 describes the purification of recombinant PDE4D expressed in *Saccharomyces Cerevisiae*. The use of recombinant native forms of PDE4D for the identification of PDE agonists or antagonists has the disadvantage that the PDE4D polypeptide does not have optimal physicochemical characteristics, especially with respect to aggregate formation. Aggregate formation can lead to protein precipitation and is known to potentially decrease the activity of enzymes, compared to the authentic oligomeric molecules. Thus, aggregation of the native forms of PDE4D polypeptides in screening assays aimed at identifying agonists or antagonists of PDE4D phosphodiesterase activity negatively affects the quality of these assays. In addition, the use of assays based on individual recombinant PDE4D isoforms has the disadvantage of potentially resulting in the identification of agonists or antagonists that are specific for a single isoform of a PDE4D only, instead of agonists or antagonists which act on all long or short form PDE4D isoforms together.

Figure 1: Specific phosphodiesterase activity for the different PDE4D isoforms D3 and D5 to D8, and the PDE4D core construct (DC).

Figure 2: Aggregate formation by different PDE4D isoforms and the core construct. In this figure, the gel filtration profiles of different isoforms of PDE4D (D3, D5, D6, D7, D8), and the core construct (DC) are shown. The first elution peak represents PDE4D aggregates. The pronounced shoulder in the elution profile of DC, where polypeptides of approx. 300,000 Dalton elute, indicates a decrease in aggregation of DC, as compared to the five isoforms of PDE4D.

Figure 3: Quantitation of aggregation of the core construct (DC) of PDE4D, and of the tubulin content of the core construct preparations. In A), the elution profile of DC is shown, with the fractions indicated on the bottom. B) shows an SDS-PAGE of the different fractions from the gel filtration. C) shows the analysis of optical densities of individual fractions corresponding to the Coomassie stained SDS-PAGE in B).

Figure 4: Differences in aggregation and tubulin content of different isoforms and the core construct of PDE. A) SDS-PAGE gels are shown for DC and five isoforms of PDE4D which were analysed as described above. B) The percentage of non-aggregated PDE4D is shown for the core construct and the isoforms D3 and D5 to D8, based on total PDE4D protein. DC has the highest percentage of non-aggregated PDE4D, as compared to the five isoforms. C) This figure shows the ratio between PDE4D and tubulin for DC and the isoforms D3 and D5 to D8, based on total PDE mass and total tubulin mass. As can be seen, the tubulin content of DC is significantly lower than that of the five PDE4D isoforms indicated.

Figure 5: Activity assay. Phosphodiesterase activity is shown both for the PDE4D core construct (DC) and PDE4D3 (D3).

Figure 6: Rolipram inhibition. Phosphodiesterase activity of the PDE4D core construct (DC) can be inhibited by Rolipram. The IC₅₀ of DC phosphodiesterase activity by Rolipram inhibition was 0.34+/- 0.06 mM.

[0013] The term "polypeptide" as used herein, refers to a polymer of amino acids, and not to a specific length. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide.

[0014] The term "physicochemical properties" as used herein refers to the propensity of polypeptides to monomer formation, oligomer formation or aggregate formation.

[0015] The terms "aggregation" and "aggregate formation" as used herein are used interchangeably and refer to the type of association of polypeptide chains which, in contrast to oligomer formation, is an irreversible process and does not follow the law of mass interaction. Aggregation progresses in a time dependent manner and does not stop when a defined number of chains have associated. Aggregation of polypeptides can lead to protein precipitation because the molecular size of the aggregate is so dramatically increased that the solubility of the molecules is impaired. Aggregation is also known to potentially decrease the activity of the enzyme as compared to authentic oligomeric molecules.

[0016] The term "oligomer formation" as used herein refers to a reversible association of polypeptide chains whereby the ratio of oligomeric proteins and monomeric proteins at a certain protein concentration and at thermodynamic equilibrium is described by the association constant.

[0017] The term "non-aggregated" refers to polypeptides that are present either as oligomers or monomers or a mixture of oligomers and monomers at equilibrium according to the law of mass interaction.

[0018] The term "improved physicochemical properties" as used herein refers to a decrease in aggregate formation of polypeptides. For the purpose of definition, aggregate formation of the polypeptides of this invention is determined by quantitation of the fractions of a gel filtration experiment in which aggregated PDE elutes, and of the fractions in which non-aggregated PDE elutes. The degree of aggregation is calculated in percent of non-aggregated PDE based on total (aggregated and non-aggregated) PDE. For the purpose of the definition of aggregation, aggregated PDE is considered to elute in fractions 2 to 4, in which aggregated molecules of at least 2 Mio Daltons elute, and non-aggregated PDE is considered to elute in fractions 5 to 11, corresponding to the fractions in which molecules of about 0.3 Mio Daltons elute, when the following conditions are used for gel filtration: 50 µl of Ni-NTA agarose purified isoform preparation are injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl₂ at a flow rate of 0.1 ml/min at 4 °C. Chromatograms are recorded at 278 nm. Starting from the elution volume onwards, the column eluate is collected as 50 µl fractions.

[0019] The term "long form PDE" as used herein refers to PDE isoforms which comprise an isoform specific N-terminal domain, linker regions LR1 and/or LR2, and upstream conserved regions UCR1 and UCR2.

[0020] The term "UCR1 start" as used herein refers to the first amino acid of the UCR1 domain of PDE4D, as annotated in SEQ ID No. 1 to 7.

[0021] The term "LF1 site" as used herein refers to the first amino acid of the splice site of PDE4D as indicated in SEQ ID No. 1-7.

[0022] The term "amino acids upstream of LF1" refers to the position of an amino acid counted from the LF-1 site towards the amino terminus of the native protein.

[0023] The term "D3" as used herein refers to the PDE4D3 isoform. The term "D4" as used herein refers to the PDE4D4 isoform. The term "D5" as used herein refers to the PDE4D5 isoform. The term "D6" as used herein refers to the PDE4D6 isoform as provided by WO02/074992. The term "D7" as used herein refers to the PDE4D7 isoform as provided by WO02/074992. The term "D8" as used herein refers to the PDE4D8 isoform as provided by WO02/074992. The polypeptide sequences of the human PDE4D isoforms D3, D4, D5, D6, D7, and D8 are shown in SEQ ID No.1 and 3-7.

[0024] The term "Ser54" as used herein refers to the Ser residue in position 54 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE polypeptides. The position corresponding to Ser54 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

[0025] The term "Ser579" as used herein refers to the Ser residue in position 579 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE polypeptides. The position corresponding to Ser579 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

[0026] The term "tubulin association" as used herein refers to the tubulin content of purified PDE preparations.

[0027] As used herein, the term "agonist" also refers to activators, and the term "antagonist" also refers to inhibitors of PDE phosphodiesterase activity.

[0028] The disadvantage of native recombinant PDE polypeptides resides in the formation of aggregates of the polypeptides, which gives rise to sub-optimal conditions for the identification of modulators of PDE phosphodiesterase activity in screening assays since aggregate formation is known to lead to polypeptide precipitation and to potentially impair activity of a polypeptide. The present invention is directed to the generation of long form PDE⁴ polypeptides with improved physicochemical properties which are particularly useful for the purpose of identifying agonists or antagonists of these enzymes.

[0029] The problem of aggregate formation of recombinant long form PDE4 polypeptides was overcome in the present invention by generating a polypeptide comprising a long form PDE4 polypeptide sequence with the exception of the PDE4A polypeptide sequence, with an amino-terminal deletion between the L71 splice site and the UCR1 start said polypeptide exhibiting decreased aggregate formation, as shown in Figures 2 and 3. These polypeptides have a specific activity that is comparable to the specific activity of the native recombinant proteins, as is shown in Figure 1. In a preferred embodiment, the invention provides a polypeptide comprising a long form PDE4 polypeptide sequence, with an amino-terminal deletion wherein the proportion of non-aggregated long form PDE4 in the total protein preparation is from 55 %, preferably from 60%, more preferably from 65%, most preferably from 68% to 100%, preferably to 90 %, more preferably to 80 %, most preferably to 70% of total protein, as determined by the quantitation of eluted fractions from a gel filtration of the long form PDE4 fractions. Quantitation is performed as follows: Equal volume aliquots of fractions from Size Exclusion Chromatography, run as hereinbefore described, are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained long form PDE4 are integrated as follows: After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE bands were integrated using a Macintosh computer and the public domain software "NIH Image", version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The integrated arbitrary units per PDE band as returned by the software reflect the relative PDE concentrations within the original pools. Integrated densities from PDE bands of fractions 2-4 are added up, giving a relative amount of aggregated PDE, PDE_{agg} (Figure 3). These fractions contain molecules of at least 2 Mio Daltons. Similarly, integrated densities from PDE bands of fractions 5-11 are added up, giving a relative amount of non-aggregated PDE, $PDE_{non-agg}$. In fractions 5-11, molecules in the range of 0.3 Mio Daltons elute. Relative amount of total PDE, PDE_{total} , is given by the sum of PDE_{agg} and $PDE_{non-agg}$. The percentage of non-aggregated PDE is given by the ratio of $PDE_{non-agg}$ and PDE_{total} , multiplied by 100.

[0030] In an even more preferred embodiment, the polypeptide sequence is a PDE4D polypeptide sequence, preferably a long form PDE4D polypeptide sequence.

[0031] Methods of generating amino-terminal deletion mutants of polypeptides are well known. Such deletion mutants can be generated by using site directed mutagenesis, e.g. by using PCR (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA).

[0032] In one embodiment of the present invention, the polypeptide comprises a PDE polypeptide sequence starting between 25 amino acids, preferably between 20 amino acids, more preferably between 15 amino acids, even more preferably between 10 amino acids, most preferably between 5 amino acids upstream of LF1 and the LF1 site.

[0033] In another embodiment the PDE long form polypeptide sequence starts at any amino acid located between the LF1 splice site and the first amino acid of the UCR1 start of the native long form PDE4 polypeptide. In the case of the most preferred embodiment of the long form PDE4 polypeptide, PDE4D, these sites are identical in all of the long form PDE4D isoforms.

[0034] In another more preferred embodiment of the present invention, the amino-terminal deletion is such that the PDE polypeptide sequence starts at the LF1 splice site of the native PDE polypeptide. This PDE polypeptide sequence is referred to as the "core construct". The core construct spans a sequence of PDE which is shared by all isoforms. The core construct sequence of PDE4D is shown in SEQ ID No. 2.

[0035] In another more preferred embodiment of the present invention, the start of the PDE polypeptide sequence is located 13 amino acids upstream of the UCR1 start of the native PDE polypeptide. The location of this position in the long form PDE4D isoforms and the core construct of PDE4D is shown in SEQ ID No. 1-7.

[0036] The basis for the generation of the above mentioned long form PDE4 polypeptide with an amino-terminal deletion and improved physicochemical characteristics can be any isoform of long form PDE4, with the exception of PDE4A. For the most preferred embodiment the long form PDE4 polypeptide sequence is an isoform of PDE4D selected from the group consisting of D3, D4, D5, D6, D7, and D8. However, the present invention also extends to further isoforms of long form PDE4D that have not yet been identified.

[0037] Also of use to the present invention are mutants of the long form PDE4 polypeptide herein before described. Of particular interest is a long form PDE⁴ polypeptide sequence comprising one or more mutations of Serine residues. Preferably, said Serines are mutated to either Alanine or Aspartic acid. In a most preferred embodiment, said Serine residues are selected from the group consisting of Ser54 and Ser579. Such Serines represent targets for phosphorylation of long form PDE⁴ polypeptides. Phosphorylation of some of these sites has been shown to activate or inhibit the phosphodiesterase activity of PDEs.

[0038] Methods of generating amino acid substitutions in polypeptides are well known to those skilled in the art of Molecular Biology, e.g. methods such as site directed mutagenesis as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA.

[0039] In another preferred embodiment of the present invention, the long form PDE⁴ polypeptide hereinbefore described exhibits decreased tubulin association, thus allowing to obtain purer long form PDE⁴ polypeptide preparations. To determine the decrease in tubulin association, the ratio of PDE molecules per tubulin molecules in the preparation of PDE polypeptide is calculated by quantitation of SDS-PAGE. Preferably, the ratio of PDE molecules per tubulin

molecules is at least 5, preferably at least 10, more preferably at least 15, even more preferably at least 20, most preferably at least 25 molecules of PDE per molecules of tubulin. The tubulin content of PDE preparations is determined as follows: Equal volume aliquots of fractions from SEC are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE and tubulin bands are integrated as described hereinbefore for PDE aggregation quantitation. Integrated densities from PDE bands of fractions 2-11 are added up, giving a relative amount of total PDE, PDE_{total} . Likewise, integrated densities from tubulin bands of fractions 2-11 are added up, giving tubulin₂₋₁₁, and thereby the relative amount of total tubulin, tubulin_{total}. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of the relative amount of total PDE4D, PDE_{tot} , and relative amount of total tubulin, tubulin_{total}, on a mass per mass basis.

[0040] The long form PDE⁴ polypeptides from which the peptide of this invention is generated by amino-terminal deletion as hereinbefore described include polypeptides that are substantially homologous or identical to the polypeptides shown in SEQ ID No. 1-7 and have, in their native form, the phosphodiesterase activity and specificity long form of PDE⁴. In particular, these long form PDE⁴ polypeptides also include the previously published polypeptide sequences of PDE4D3 to D8 as defined in U50159 (D3), L20969 (D4), S:1059276 (D5) and the sequences for D6, D7 and D8 as disclosed in WO02/074992. As used herein, two polypeptides (or a region of the polypeptides) are substantially homologous or identical when the amino acid sequences are at least 45-55%, typically at least 70-75%, more typically at least 80-85%, and most typically greater than 90% or more homologous or identical. To determine the percent homology or identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid molecule). The amino acid residues or nucleotides at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules are homologous at that position. As used herein, amino acid "homology" is equivalent to amino acid "identity". The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, percent homology equals the number of identical positions/total number of positions times 100). The long form PDE⁴ polypeptide sequences of the present invention are preferably mammalian, most preferably human. The present invention also includes variants of the polypeptides hereinbefore described with deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, Science 247 (1990), 1306-1310, wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

[0041] The present invention provides polynucleotides that comprise polynucleotide sequences that encode the long form PDE⁴ polypeptide sequences hereinbefore described. As an example for a sequence comprised in such a polynucleotide, the polynucleotide sequence encoding the "core construct" of PDE4D is shown in SEQ ID No. 8. However, the present invention relates to all polynucleotides comprising polynucleotide sequences encoding any of the PDE polypeptide sequences hereinbefore described.

[0042] The present invention provides an expression vector or virus comprising a polynucleotide sequence that encodes the long form PDE⁴ polypeptide sequences hereinbefore described and which is capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell. The invention also provides a prokaryotic or eukaryotic host cell transformed or infected with such an expression vector or virus. The invention further provides a process for the production of such a polypeptide comprising culturing the host cell expressing the long form PDE⁴ polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells. In a preferred embodiment, the virus used for the expression of a modified long form PDE⁴ polypeptide is a recombinant baculovirus. Methods of generating recombinant baculoviruses for expression of recombinant proteins are well known to the man skilled in the art. Preferably, the recombinant baculovirus is based on pFASTBac as the transfer vector. The host cell of a preferred embodiment of the invention is an insect cell. Insect cells that can be infected with recombinant baculoviruses for the purpose of expressing recombinant proteins are well known in the art. The preferred process for the production of a modified long form PDE⁴ polypeptide is a process comprising culturing the insect cell expressing the long form PDE⁴ polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the insect cell. To facilitate purification, the long form PDE⁴ polypeptide may also comprise a tag. Different types of such tags and the methods to join them with polynucleotide sequences encoding a polypeptide are well known to the person skilled in the art, e.g. myc-tag, FLAG-tag, six Histidine tags (hereinafter referred to as 6xHis tag). A review on tagging of proteins can be found e.g. in Fritze and Anderson, Methods in Enzymology (2000) 327, 3-16. It is well known that such tags may be joined to either side of the polypeptide. Some tags are known which may also be removed enzymatically from the purified polypeptide, e.g. FLAG-tag fused to the amino-terminus of a protein may be removed by digestion with bovine enterokinase (Hopp, T.P.(1988) Biotechnology, 6, 1204-1210). In a preferred embodiment the long form PDE⁴ polypeptides hereinbefore described are linked at their C-terminus to a 6xHis tag. The use of such 6xHis tags for ease of purification of recombinant polypeptides, and methods of purification of 6xHis tagged polypeptides are well known (e.g. in Sisk et al. (1994) J. Virol.

68, 766, and Petty, K.J. (1996) in: Ausubel, F.M. et al., eds. Current Protocols in Molecular Biology, Vol. 2, New York: John Wiley and Sons).

[0043] In a preferred embodiment, the polypeptide of the present invention is purified. As used herein, a polypeptide is said to be "purified" when it is substantially free of cellular material when it is isolated from recombinant and non-recombinant cells. A polypeptide, however, can be joined to another polypeptide with which it is not normally associated in a cell (e.g. in a "fusion protein") and still be "purified". Preferably, the polypeptides of the present invention are purified. The polypeptides of the invention can be purified to homogeneity. It is understood, however, that preparations in which the polypeptide is not purified to homogeneity are useful. The critical feature is that the preparation allows for the desired function of the polypeptide, even in the presence of considerable amounts of other components. Thus, the invention encompasses various degrees of purity. In one embodiment, the language "substantially free of cellular material" includes preparations of the polypeptide having less than about 30% (by dry weight) other proteins (i.e., contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When a polypeptide is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the polypeptide preparation.

[0044] The present invention also provides for a use of the polypeptides hereinbefore described for identifying an agonist or antagonist of phosphodiesterase activity.

[0045] The polypeptides of the present invention can be used for a process of identifying and obtaining a drug candidate for therapy of a vascular disorder, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the above mentioned polypeptides. Different methods for measuring phosphodiesterase activity are known, eg. from Owens et al., Biochem. J. (1997) 326, 53-60. Such a drug candidate may be a compound that is either an agonist or an antagonist of the phosphodiesterase activity of PDE.

[0046] The present invention also provides a screening assay for the identification of agonists or antagonists of phosphodiesterase activity comprising at least one of the above described polypeptides. In addition, the polypeptides hereinbefore described can be used for crystallization. Crystals of the polypeptides are useful for assaying the interaction of compounds with phosphodiesterases.

[0047] Furthermore, the present invention provides a kit for identifying agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides hereinbefore described, and a radioactive or IMKP - FP phosphodiesterase kit.

[0048] The invention will be further described by the following non-limiting examples.

Examples

[0049] In the following the term "isoform" relates to the natural PDE4D splice variants D3 and D5-D8 and the invented core variant, DC.

Cloning of the PDE4D isoforms 3-8 and the core construct

Core construct:

[0050] A cDNA encoding the core fragment that is common for all the PDE4D isoforms 3-8 starting with the amino acid sequence FDV carboxyterminal to the LF1 splice site was generated by PCR using a 5' oligonucleotide with a HindIII cloning site (gatgaattcaagcttttgatgtggacaatggcaca) introducing two additional amino acids (K and L) in front of the FDV sequence. At the 3' end a set of primers was used that either generated the native sequence (gtgatatctcattatcacgtgtcaggagaacgatcatctatgaca) or added a sequence encoding 6xHis residues (gtgatatctcattatcaa tgggatggatggatggcgtgtcaggagaacgatcatctatgac) to enable rapid purification of the recombinant proteins. The cDNA encoding the core construct was cloned as a EcoRI-EcoRV fragment into the expression vector pENTR™1a (GIBCO/BRL)

Isoforms (except core construct):

The DNA fragments encoding the isoform specific N-termini were generated by using synthetic oligonucleotides with terminal restriction enzyme sites for EcoRI and HindIII incorporated for directional cloning. These isoform specific DNA fragments were fused to the core construct sequence via the HindIII site introducing two additional amino acid residues (K and L). The integrity of the clones was confirmed by DNA sequencing prior to expression.

Expression of the PDE4D isoforms and the core construct in insect cells

[0051] The cDNAs were cloned into the pFASTBAC1 vector (Life Technologies, Inc) for expression in insect cells and the products were confirmed by sequencing. After recombination into the baculovirus genome the purified viral DNAs were transformed into the insect cells. Sf9 cells were cultured at 27°C in TC100 medium (BioWhittaker) with 5% (v/v)

fetal calf serum. Virus stocks were generated with a titer of 1.5×10^9 pfu/ml. For large scale production of the isoforms 1-24 L fermentations of Sf9 cells were infected with a MOI of 1.

[0052] In one example, the 6xHis tagged PDE4D polypeptides DC, D3, D5, D6, D7 and D8 were produced in Sf9 cells in 1L spinner flasks using SF1 medium in the absence of serum. Infected cells were harvested 3 days after infection with the recombinant baculoviruses.

[0053] In another example, the PDE4D core construct DC was produced in a 24 L Airlifter Fermenter with 15 L medium (SF1 with 0% serum), 0.15 L lipids and 9 L Sf9 cells. During the entire fermentation procedure the cells were cultured at pH 6.2, $27.0 \pm 0.2^\circ\text{C}$ and a $p\text{O}_2$ of $30.0 \pm 0.5\%$. Cells were grown for 3 days. The cell number at infection was 2.3×10^6 cells/ml. Cells were infected with 450 ml recombinant baculovirus. Cells were harvested at 68h post infection and the cell pellet as well as the concentrated supernatant stored at -80°C until further processing.

Purification of 6xHis tagged PDE4D isoforms

[0054] Sf9 cells from 1 liter culture broth, overexpressing the respective isoform, were resuspended on ice in 50 ml 50 mM HEPES pH 7.8, 300 mM NaCl 10 mM imidazole, 1 mM DTT, supplemented with protease inhibitors (one protease inhibitor cocktail tablet "complete, EDTA-free"; Roche). Opening of cells was performed by use of a 50 ml Dounce homogenizator and the homogeneous mixture was centrifuged for 1 hour at $70'000\text{ g}$ and 4°C (Kontron TFT 45.94 rotor at $30'000\text{ rpm}$). The supernate was filtrated through a filter with a pore size of $1.2\text{ }\mu\text{m}$ (Minisart; Sartorius, Germany) and then applied to a 6 ml Ni-NTA agarose column at 2 ml/min. After equilibration with 50 mM HEPES pH 7.8, 300 mM NaCl, 10 mM imidazole, protein was eluted with a linear 30 ml gradient from 10 to 230 mM imidazole in the same buffer. Fractions containing the PDE4D isoform as analyzed by Coomassie stained SDS-PAGE were pooled and stored frozen at -80°C . Fresh NiNTA agarose material was used for every different PDE4D isoform preparation in order to prevent cross contamination of isoforms.

Specific activities of 6xHis tagged PDE4D isoforms

[0055] Relative concentrations of Ni-NTA agarose purified isoform preparations were estimated by SDS-PAGE. Equal volume amounts of isoform preparations were applied to a gradient gel (4-12% NuPage; Invitrogen). After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE4D bands were integrated using a Macintosh computer and the public domain software "NIH Image", version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The integrated arbitrary units per PDE4D band as returned by the software reflect the relative PDE4D concentrations within the original pools (Figure 1, "amount"). Identities of PDE4D and tubulin bands had been verified by independent SDS-PAGE, excision of corresponding bands, trypsin cleavage and identification of tryptic peptides by MALDI-MS.

[0056] Activities of equal volume amounts of 10^6 -fold diluted purified isoforms were determined by use of a commercial radioactive phosphodiesterase assay (cAMP-dependent phosphodiesterase [^3H] assay; Amersham Pharmacia Biotech), following the instructions of the manufacturer. The obtained arbitrary activity units reflect the relative PDE4D activities within the original pools (Figure 1, "activity").

[0057] Relative specific activities of PDE4D isoforms were calculated by dividing relative activity values by relative concentration values (Figure 1, "specific activity").

Qualitative investigation of aggregation by size exclusion chromatography (SEC)

[0058] $50\text{ }\mu\text{l}$ of Ni-NTA agarose purified isoform preparation was injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl_2 at a flow rate of 0.1 ml/min at 4°C . Chromatograms were recorded at 278 nm. Starting from the elution volume, the column eluate was collected as $50\text{ }\mu\text{l}$ fractions (Figure 2).

Quantification of aggregates and tubulin content

[0059] Equal volume aliquots of fractions from SEC were analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE4D and tubulin bands were integrated as described above. Integrated densities from PDE4D bands of fractions 2-4 were added up, giving a relative amount of aggregated PDE4D, PDE_{agg} (Figure 3). Similarly, integrated densities from PDE4D bands of fractions 5-11 were added up, giving a relative amount of non-aggregated PDE4D, $\text{PDE}_{\text{non-agg}}$. Relative amount of total PDE4D, $\text{PDE}_{\text{total}}$, is given by the sum of PDE_{agg} and $\text{PDE}_{\text{non-agg}}$. The percentage of non-aggregated PDE is given by the ratio of $\text{PDE}_{\text{non-agg}}$ and $\text{PDE}_{\text{total}}$, multiplied by 100 (Figure 4B).

[0060] Similarly, integrated densities from tubulin bands of fractions 2-11 were added up, giving tubulin₂₋₁₁, and thereby

the relative amount of total tubulin, $\text{tubulin}_{\text{total}}$. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of relative amount of total PDE4D, PDE_{tot} , and relative amount of total tubulin, $\text{tubulin}_{\text{total}}$.

Activity assay and inhibition of phosphodiesterase activity

[0061] An IMAP FP-Assay was used for the determination of phosphodiesterase activity. The phosphodiesterase activity of the core construct and PDE4D3 was measured using the HEFP Phosphodiesterase Assay Kit (Molecular Devices). 2 μl of PDE4D3 or PDE4D core construct, 2 μl of cAMP (to a final concentration of 40 nM) and 1 μl of test substance or carrier were incubated for 45 min on a shaker. 12 μl of Binding Solution provided by the kit (with beads diluted 1:320) were added, and the reaction mixture incubated on a shaker for 2 hours. Fluorescence polarisation of the samples was measured in a Packard BioScience Fusion a-FP HT using as an emission filter a Polarizer 535, and as an excitation filter, Fluorescein 485/20. Inhibition of the phosphodiesterase activity of the PDE4D core construct was determined using Rolipram as inhibitor, and using PDE4D core construct at 30 ng/ml.

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	Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr	
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	Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His	
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	Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu	
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	Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala	
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	Ala Ile Phe Ala Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser	
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	Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn	
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	Asp Ser Ser Val Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu	
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	Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln	
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	Arg Gln Ser Leu Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp	
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Thr Lys Lys Val Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser
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Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro

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Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His

20 50 55 60

Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp

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5	Leu	Ser	Pro	Lys	Ser	Met	Ser	Arg	Asn	Ser	Ser	Ile	Ala	Ser	Asp	Ile
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10	His	Gly	Asp	Asp	Leu	Ile	Val	Thr	Pro	Phe	Ala	Gln	Val	Leu	Ala	Ser
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15	Leu	Arg	Thr	Val	Arg	Asn	Asn	Phe	Ala	Ala	Leu	Thr	Asn	Leu	Gln	Asp
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20	Arg	Ala	Pro	Ser	Lys	Arg	Ser	Pro	Met	Cys	Asn	Gln	Pro	Ser	Ile	Asn
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25	Lys	Ala	Thr	Ile	Thr	Glu	Glu	Ala	Tyr	Gln	Lys	Leu	Ala	Ser	Glu	Thr
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30	Leu	Glu	Glu	Leu	Asp	Trp	Cys	Leu	Asp	Gln	Leu	Glu	Thr	Leu	Gln	Thr
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40	Asn	Arg	Glu	Leu	Thr	His	Leu	Ser	Glu	Met	Ser	Arg	Ser	Gly	Asn	Gln
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45	Val	Ser	Glu	Phe	Ile	Ser	Asn	Thr	Phe	Leu	Asp	Lys	Gln	His	Glu	Val
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55	Met	Ser	Gln	Ile	Ser	Gly	Val	Lys	Lys	Leu	Met	His	Ser	Ser	Ser	Leu
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	Thr	Asn	Ser	Ser	Ile	Pro	Arg	Phe	Gly	Val	Lys	Thr	Glu	Gln	Glu	Asp

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15	His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro		
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20	Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His		
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	340	345	350
25	Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln		
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30	Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr		
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35	Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp Val		
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40	Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His Leu		
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45	Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln		
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50	Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp		
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55	Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala Asp		

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5	Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val Leu		
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10	Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met Val		
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15	His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg		
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25	Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn		
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30	Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His		
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35	Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp		
	565	570	575
40	Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile		
	580	585	590
45	Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln		
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50	Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly		
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55	Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr		
	625	630	635 640
	Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr		

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	645	650	655
5	Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu		
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15 35 40 45

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	Ser	Glu	Asn	Ile	Gln	Arg	Pro	Thr	Ser	Leu	Pro	Leu	Lys	Ile	Leu	Pro
10	65					70					75					80
	Leu	Ile	Ala	Ile	Thr	Ser	Ala	Glu	Ser	Ser	Gly	Lys	Leu	Phe	Asp	Val
15						85					90				95	
	Asp	Asn	Gly	Thr	Ser	Ala	Gly	Arg	Ser	Pro	Leu	Asp	Pro	Met	Thr	Ser
20						100					105				110	
	Pro	Gly	Ser	Gly	Leu	Ile	Leu	Gln	Ala	Asn	Phe	Val	His	Ser	Gln	Arg
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	Arg	Glu	Ser	Phe	Leu	Tyr	Arg	Ser	Asp	Ser	Asp	Tyr	Asp	Leu	Ser	Pro
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	Lys	Ser	Met	Ser	Arg	Asn	Ser	Ser	Ile	Ala	Ser	Asp	Ile	His	Gly	Asp
35						145					150				155	160
	Asp	Leu	Ile	Val	Thr	Pro	Phe	Ala	Gln	Val	Leu	Ala	Ser	Leu	Arg	Thr
40						165					170				175	
	Val	Arg	Asn	Asn	Phe	Ala	Ala	Leu	Thr	Asn	Leu	Gln	Asp	Arg	Ala	Pro
45						180					185				190	
	Ser	Lys	Arg	Ser	Pro	Met	Cys	Asn	Gln	Pro	Ser	Ile	Asn	Lys	Ala	Thr
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	Ile	Thr	Glu	Glu	Ala	Tyr	Gln	Lys	Leu	Ala	Ser	Glu	Thr	Leu	Glu	Glu
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	Leu	Asp	Trp	Cys	Leu	Asp	Gln	Leu	Glu	Thr	Leu	Gln	Thr	Arg	His	Ser
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	Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu	
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10		
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	275	280 285
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	Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln	
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	Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser	
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	Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala	
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	Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val Phe Arg Ile	
	340	345 350
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	Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met His Thr Ile	
	355	360 365
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	Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr	
	370	375 380
45		
	Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val	
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	Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His	
	405	410 415
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	Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu	
	420	425 430

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				435					440					445		
5																
	Gly	Val	Ser	Asn	Gln	Phe	Leu	Ile	Asn	Thr	Asn	Ser	Glu	Leu	Ala	Leu
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	Met	Tyr	Asn	Asp	Ser	Ser	Val	Leu	Glu	Asn	His	His	Leu	Ala	Val	Gly
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	Phe	Lys	Leu	Leu	Gln	Glu	Glu	Asn	Cys	Asp	Ile	Phe	Gln	Asn	Leu	Thr
							485				490			495		
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	Lys	Lys	Gln	Arg	Gln	Ser	Leu	Arg	Lys	Met	Val	Ile	Asp	Ile	Val	Leu
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	Asp	Leu	Ser	Asn	Pro	Thr	Lys	Pro	Leu	Gln	Leu	Tyr	Arg	Gln	Trp	Thr
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	Asp	Arg	Ile	Met	Glu	Glu	Phe	Phe	Arg	Gln	Gly	Asp	Arg	Glu	Arg	Glu
							580			585				590		
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	Arg	Gly	Met	Glu	Ile	Ser	Pro	Met	Cys	Asp	Lys	His	Asn	Ala	Ser	Val
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	Glu	Lys	Ser	Gln	Val	Gly	Phe	Ile	Asp	Tyr	Ile	Val	His	Pro	Leu	Trp
							610			615				620		

Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp

625 630 635 640

Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser

645 650 655

Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr

660 665 670

Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp

675 680 685

Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser

690 695 700

Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro

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Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr

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 Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser Gly Leu Ile Leu
 35 40 45
 Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg
 50 55 60
 Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met Ser Arg Asn Ser
 65 70 75 80
 Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile Val Thr Pro Phe
 85 90 95
 Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn Asn Phe Ala Ala
 100 105 110
 Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg Ser Pro Met Cys
 115 120 125
 Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln
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 Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln
 145 150 155 160
 Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala Ser Asn
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5	Ser	Arg	Ser	Gly	Asn	Gln	Val	Ser	Glu	Phe	Ile	Ser	Asn	Thr	Phe	Leu
						195				200					205	
10	Asp	Lys	Gln	His	Glu	Val	Glu	Ile	Pro	Ser	Pro	Thr	Gln	Lys	Glu	Lys
						210				215					220	
15	Glu	Lys	Lys	Lys	Arg	Pro	Met	Ser	Gln	Ile	Ser	Gly	Val	Lys	Lys	Leu
						225				230					235	240
20	Met	His	Ser	Ser	Ser	Leu	Thr	Asn	Ser	Ser	Ile	Pro	Arg	Phe	Gly	Val
						245					250				255	
25	Lys	Thr	Glu	Gln	Glu	Asp	Val	Leu	Ala	Lys	Glu	Leu	Glu	Asp	Val	Asn
						260					265				270	
30	Lys	Trp	Gly	Leu	His	Val	Phe	Arg	Ile	Ala	Glu	Leu	Ser	Gly	Asn	Arg
						275					280				285	
35	Pro	Leu	Thr	Val	Ile	Met	His	Thr	Ile	Phe	Gln	Glu	Arg	Asp	Leu	Leu
						290					295				300	
40	Lys	Thr	Phe	Lys	Ile	Pro	Val	Asp	Thr	Leu	Ile	Thr	Tyr	Leu	Met	Thr
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	Leu	Glu	Asp	His	Tyr	His	Ala	Asp	Val	Ala	Tyr	His	Asn	Asn	Ile	His
45						325					330				335	
	Ala	Ala	Asp	Val	Val	Gln	Ser	Thr	His	Val	Leu	Leu	Ser	Thr	Pro	Ala
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	Leu	Glu	Ala	Val	Phe	Thr	Asp	Leu	Glu	Ile	Leu	Ala	Ala	Ile	Phe	Ala
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Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln Phe Leu
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 Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu
 420 425 430
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 Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser Lys His
 435 440 445
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 Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val
 450 455 460
 30
 Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln
 465 470 475 480
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 Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys
 485 490 495
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 Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe
 500 505 510
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 Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro
 515 520 525
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	245 250 255
15	Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala
	260 265 270
20	Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu
	275 280 285
25	Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala
	290 295 300
30	Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His Leu Ser
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35	Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser Asn Thr
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40	Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr Gln Lys
	340 345 350
45	Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly Val Lys
	355 360 365
50	Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro Arg Phe
	370 375 380
55	Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu Glu Asp
	385 390 395 400

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						420				425					430	
10																
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10 Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln
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	Glu	Ile	Pro	Leu	Asp	Glu	Gln	Val	Glu	Glu	Glu	Ala	Val	Gly	Glu	Glu
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	Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His
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	Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu
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	Met Ser Lys His Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu
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 15 Met Glu Glu Phe Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met
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 20 Glu Ile Ser Pro Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser
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 25 Gln Val Gly Phe Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp
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 30 Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp Thr Leu Glu
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 35 Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro
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 40 Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe
 660 665 670
 45 Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys
 675 680 685
 50 Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys
 690 695 700
 55 Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu
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Gln Val Glu Glu Glu Ala Val Gly Glu Glu Glu Glu Ser Gln Pro Glu

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Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr

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10

20

25

30

Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro

15

35

40

45

Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His

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Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp

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10	His	Gly	Asp	Asp	Leu	Ile	Val	Thr	Pro	Phe	Ala	Gln	Val	Leu	Ala	Ser
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15	Leu	Arg	Thr	Val	Arg	Asn	Asn	Phe	Ala	Ala	Leu	Thr	Asn	Leu	Gln	Asp
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55	Met	Ser	Gln	Ile	Ser	Gly	Val	Lys	Lys	Leu	Met	His	Ser	Ser	Ser	Leu
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	Thr	Asn	Ser	Ser	Ile	Pro	Arg	Phe	Gly	Val	Lys	Thr	Glu	Gln	Glu	Asp

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10	Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met		
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15	His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro		
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20	Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His		
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	340	345	350
25	Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln		
	355	360	365
30	Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr		
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35	Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp Val		
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40	Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His Leu		
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45	Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln		
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50	Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp		
	435	440	445
55	Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala Asp		

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	450	455	460	
5	Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val Leu			
	465	470	475	480
10	Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met Val			
		485	490	495
15	His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg			
		500	505	510
20	Gln Trp Thr Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp Arg			
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25	Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn			
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30	Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His			
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35	Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp			
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40	Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile			
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50	Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly			
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55	Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr			
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	Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr			640

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	645	650	655
5	Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu		
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Met Lys Arg Asn Thr Cys Asp Leu Leu Ser Arg Ser Lys Ser Ala Ser

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Glu Glu Thr Leu His Ser Ser Asn Glu Glu Glu Asp Pro Phe Arg Gly

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Met Glu Pro Tyr Leu Val Arg Arg Leu Ser Cys Arg Asn Ile Gln Leu

35 40 45

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Pro Pro Leu Ala Phe Arg Gln Leu Glu Gln Ala Asp Leu Lys Ser Glu
5 50 55 60
Ser Glu Asn Ile Gln Arg Pro Thr Ser Leu Pro Leu Lys Ile Leu Pro
65 70 75 80
10 Leu Ile Ala Ile Thr Ser Ala Glu Ser Ser Gly Lys Leu Phe Asp Val
85 90 95
15 Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser
100 105 110
20 Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg
115 120 125
25 Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro
130 135 140
30 Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp
145 150 155 160
35 Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr
165 170 175
Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro
40 180 185 190
Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr
45 195 200 205
Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu
50 210 215 220
Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser
225 230 235 240
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	Leu	Thr	His	Leu	Ser	Glu	Met	Ser	Arg	Ser	Gly	Asn	Gln	Val	Ser	Glu
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	Phe	Ile	Ser	Asn	Thr	Phe	Leu	Asp	Lys	Gln	His	Glu	Val	Glu	Ile	Pro
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	Ser	Pro	Thr	Gln	Lys	Glu	Lys	Glu	Lys	Lys	Lys	Arg	Pro	Met	Ser	Gln
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	Ile	Ser	Gly	Val	Lys	Lys	Leu	Met	His	Ser	Ser	Ser	Leu	Thr	Asn	Ser
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	Phe	Gln	Glu	Arg	Asp	Leu	Leu	Lys	Thr	Phe	Lys	Ile	Pro	Val	Asp	Thr
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	Val	Leu	Leu	Ser	Thr	Pro	Ala	Leu	Glu	Ala	Val	Phe	Thr	Asp	Leu	Glu

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	Ile	Leu	Ala	Ala	Ile	Phe	Ala	Ser	Ala	Ile	His	Asp	Val	Asp	His	Pro
5				435					440					445		
	Gly	Val	Ser	Asn	Gln	Phe	Leu	Ile	Asn	Thr	Asn	Ser	Glu	Leu	Ala	Leu
10				450					455					460		
	Met	Tyr	Asn	Asp	Ser	Ser	Val	Leu	Glu	Asn	His	His	Leu	Ala	Val	Gly
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	Phe	Lys	Leu	Leu	Gln	Glu	Glu	Asn	Cys	Asp	Ile	Phe	Gln	Asn	Leu	Thr
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	Lys	Lys	Gln	Arg	Gln	Ser	Leu	Arg	Lys	Met	Val	Ile	Asp	Ile	Val	Leu
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	Ala	Thr	Asp	Met	Ser	Lys	His	Met	Asn	Leu	Leu	Ala	Asp	Leu	Lys	Thr
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	Met	Val	Glu	Thr	Lys	Lys	Val	Thr	Ser	Ser	Gly	Val	Leu	Leu	Leu	Asp
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	Asn	Tyr	Ser	Asp	Arg	Ile	Gln	Val	Leu	Gln	Asn	Met	Val	His	Cys	Ala
40				545					550					555		560
	Asp	Leu	Ser	Asn	Pro	Thr	Lys	Pro	Leu	Gln	Leu	Tyr	Arg	Gln	Trp	Thr
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	Asp	Arg	Ile	Met	Glu	Glu	Phe	Phe	Arg	Gln	Gly	Asp	Arg	Glu	Arg	Glu
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	Arg	Gly	Met	Glu	Ile	Ser	Pro	Met	Cys	Asp	Lys	His	Asn	Ala	Ser	Val
55				595					600						605	
	Glu	Lys	Ser	Gln	Val	Gly	Phe	Ile	Asp	Tyr	Ile	Val	His	Pro	Leu	Trp
				610					615						620	

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Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp
 5 625 630 635 640
 Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser
 10 645 650 655
 Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr
 15 660 665 670
 Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp
 20 675 680 685
 Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser
 25 690 695 700
 Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro
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	Thr	Ala	Glu	Ala	Lys	Leu	Phe	Asp	Val	Asp	Asn	Gly	Thr	Ser	Ala	Gly
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	Arg	Ser	Pro	Leu	Asp	Pro	Met	Thr	Ser	Pro	Gly	Ser	Gly	Leu	Ile	Leu
15					35					40					45	
	Gln	Ala	Asn	Phe	Val	His	Ser	Gln	Arg	Arg	Glu	Ser	Phe	Leu	Tyr	Arg
20					50					55					60	
	Ser	Asp	Ser	Asp	Tyr	Asp	Leu	Ser	Pro	Lys	Ser	Met	Ser	Arg	Asn	Ser
25					65					70					75	
	Ser	Ile	Ala	Ser	Asp	Ile	His	Gly	Asp	Asp	Leu	Ile	Val	Thr	Pro	Phe
30										85					90	
	Ala	Gln	Val	Leu	Ala	Ser	Leu	Arg	Thr	Val	Arg	Asn	Asn	Phe	Ala	Ala
35										100					105	
	Leu	Thr	Asn	Leu	Gln	Asp	Arg	Ala	Pro	Ser	Lys	Arg	Ser	Pro	Met	Cys
40										115					120	
	Asn	Gln	Pro	Ser	Ile	Asn	Lys	Ala	Thr	Ile	Thr	Glu	Glu	Ala	Tyr	Gln
45										130					135	
	Lys	Leu	Ala	Ser	Glu	Thr	Leu	Glu	Glu	Leu	Asp	Trp	Cys	Leu	Asp	Gln
50										145					150	
	Leu	Glu	Thr	Leu	Gln	Thr	Arg	His	Ser	Val	Ser	Glu	Met	Ala	Ser	Asn
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5	Ser	Arg	Ser	Gly	Asn	Gln	Val	Ser	Glu	Phe	Ile	Ser	Asn	Thr	Phe	Leu
						195				200					205	
10	Asp	Lys	Gln	His	Glu	Val	Glu	Ile	Pro	Ser	Pro	Thr	Gln	Lys	Glu	Lys
						210				215					220	
15	Glu	Lys	Lys	Lys	Arg	Pro	Met	Ser	Gln	Ile	Ser	Gly	Val	Lys	Lys	Leu
						225				230					235	
20	Met	His	Ser	Ser	Ser	Leu	Thr	Asn	Ser	Ser	Ile	Pro	Arg	Phe	Gly	Val
						245				250					255	
25	Lys	Thr	Glu	Gln	Glu	Asp	Val	Leu	Ala	Lys	Glu	Leu	Glu	Asp	Val	Asn
						260				265					270	
30	Lys	Trp	Gly	Leu	His	Val	Phe	Arg	Ile	Ala	Glu	Leu	Ser	Gly	Asn	Arg
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35	Pro	Leu	Thr	Val	Ile	Met	His	Thr	Ile	Phe	Gln	Glu	Arg	Asp	Leu	Leu
						290				295					300	
40	Lys	Thr	Phe	Lys	Ile	Pro	Val	Asp	Thr	Leu	Ile	Thr	Tyr	Leu	Met	Thr
						305				310					315	
	Leu	Glu	Asp	His	Tyr	His	Ala	Asp	Val	Ala	Tyr	His	Asn	Asn	Ile	His
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	Ala	Ala	Asp	Val	Val	Gln	Ser	Thr	His	Val	Leu	Leu	Ser	Thr	Pro	Ala
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	Leu	Glu	Ala	Val	Phe	Thr	Asp	Leu	Glu	Ile	Leu	Ala	Ala	Ile	Phe	Ala
55						355				360					365	

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Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln Phe Leu
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 5 Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn Asp Ser Ser Val
 385 390 395 400
 10 Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Glu
 405 410 415
 15 Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu
 420 425 430
 20 Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser Lys His
 435 440 445
 25 Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val
 450 455 460
 30 Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln
 465 470 475 480
 35 Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys
 485 490 495
 40 Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe
 500 505 510
 45 Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro
 515 520 525
 50 Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser Gln Val Gly Phe
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 55 Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp Ala Asp Leu Val
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	Trp	Tyr	Gln	Ser	Thr	Ile	Pro	Gln	Ser	Pro	Ser	Pro	Ala	Pro	Asp	Asp
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	Pro	Glu	Glu	Gly	Arg	Gln	Gly	Gln	Thr	Glu	Lys	Phe	Gln	Phe	Glu	Leu
15						595					600					605
	Thr	Leu	Glu	Glu	Asp	Gly	Glu	Ser	Asp	Thr	Glu	Lys	Asp	Ser	Gly	Ser
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	Gln	Val	Glu	Glu	Asp	Thr	Ser	Cys	Ser	Asp	Ser	Lys	Thr	Leu	Cys	Thr
25						625					630					635
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	Gln	Asp	Ser	Glu	Ser	Thr	Glu	Ile	Pro	Leu	Asp	Glu	Gln	Val	Glu	Glu
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	Glu	Ala	Val	Gly	Glu	Glu	Glu	Glu	Ser	Gln	Pro	Glu	Ala	Cys	Val	Ile
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Gly Ser Asp Ser Ala Gly Gly Ala Thr Leu Lys Ala Pro Lys His Leu
20 25 30
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Arg Leu Leu His Pro His His His Leu Pro Pro Pro Pro Pro Pro Ser
50 55 60
Pro Gln Pro Gln Pro Gln Cys Pro Leu Gln Pro Pro Pro Pro Pro Pro
65 70 75 80
Leu Pro Pro Pro Pro Pro Pro Pro Gly Ala Ala Arg Gly Arg Tyr Ala
85 90 95
Ser Ser Gly Ala Thr Gly Arg Val Arg His Arg Gly Tyr Ser Asp Thr
100 105 110
Glu Arg Tyr Leu Tyr Cys Arg Ala Met Asp Arg Thr Ser Tyr Ala Val
115 120 125
Glu Thr Gly His Arg Pro Gly Leu Lys Lys Ser Arg Met Ser Trp Pro
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Ser Ser Phe Gln Gly Leu Arg Arg Phe Asp Val Asp Asn Gly Thr Ser
145 150 155 160
Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser Gly Leu
165 170 175
Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser Phe Leu
180 185 190
Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met Ser Arg
195 200 205

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Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile Val Thr
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5 Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn Asn Phe
225 230 235 240
10 Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg Ser Pro
245 250 255
15 Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala
260 265 270
20 Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu
275 280 285
25 Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala
290 295 300
30 Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His Leu Ser
305 310 315 320
Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser Asn Thr
35 325 330 335
Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr Gln Lys
40 340 345 350
Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly Val Lys
45 355 360 365
Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro Arg Phe
50 370 375 380
Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu Glu Asp
385 390 395 400
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5	Asn	Arg	Pro	Leu	Thr	Val	Ile	Met	His	Thr	Ile	Phe	Gln	Glu	Arg	Asp
						420					425				430	
10	Leu	Leu	Lys	Thr	Phe	Lys	Ile	Pro	Val	Asp	Thr	Leu	Ile	Thr	Tyr	Leu
						435					440				445	
15	Met	Thr	Leu	Glu	Asp	His	Tyr	His	Ala	Asp	Val	Ala	Tyr	His	Asn	Asn
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Claims

- 5 1. A polypeptide comprising a long form PDE4 polypeptide sequence with an amino-terminal deletion, wherein the polypeptide sequence starts at any amino acid located between the LF1 splice site and the first amino acid of the UCR1 start of the native long form PDE4 polypeptide, said polypeptide exhibiting decreased aggregate formation.
2. The polypeptide of claim 1 wherein said PDE4 polypeptide sequence is a PDE4D polypeptide sequence.
- 10 3. The polypeptide of any of claims 1 to 2, wherein the polypeptide sequence starts at the LF1 splice site of the native PDE4 polypeptide.
4. The polypeptide of any of claims 1 to 3, wherein the PDE4 polypeptide sequence start is located 13 amino acids upstream of the UCR1 start of the native PDE4 polypeptide.
- 15 5. The polypeptide of any of claims 2 to 4 wherein the PDE4D polypeptide sequence is a sequence of an isoform selected from the group consisting of D3, D4, D5, D6, D7, and D8.
6. The polypeptide of claims 1 to 5 wherein said polypeptide comprises one or more mutations of Serine residues.
- 20 7. The polypeptide of claim 6 wherein said Serine residues are mutated to either Alanine or Aspartic acid.
8. The polypeptide of claims 6 or 7 wherein said Serine residues are selected from the group consisting of Ser residues corresponding to Ser54 and Ser579 in Seq ID No. 1.
- 25 9. The polypeptide of any one of claims 1 to 5 wherein said polypeptide exhibits decreased tubulin association.
10. A polynucleotide sequence encoding a polypeptide as claimed in claims 1 to 9.
- 30 11. An expression vector or virus comprising a polynucleotide sequence as claimed in claim 10 and being capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell.
12. A virus as claimed in claim 11 wherein the virus is a recombinant baculovirus.
- 35 13. A prokaryotic or eukaryotic host cell transformed or infected with the expression vector or virus of claims 11 or 12.
14. A host cell as claimed in claim 13, wherein the host cell is an insect cell.
- 40 15. A process for the production of a polypeptide as claimed in claims 1 to 9, comprising culturing the host cell of claims 13 or 14 in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells.
16. A process for the production of a polypeptide as claimed in claim 15 wherein the host cell is an insect cell.
17. A polypeptide according to claims 1 to 9 produced by the process of claims 15 or 16.
- 45 18. A screening assay for the identification of agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 9.
19. A process for identifying a drug candidate for therapy of an inflammatory disease, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the polypeptides claimed in claims 1 to 9.
- 50 20. Use of a polypeptide of claims 1 to 9 for identifying an agonist or antagonist of phosphodiesterase activity.
21. Use of a polypeptide of claims 1 to 9 for crystallization.
- 55 22. A kit for identifying agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 9 and a radioactive or IMAP-FP phosphodiesterase kit.

Patentansprüche

- 5 1. Polypeptid, umfassend eine Sequenz der langen Form des PDE4-Polypeptids mit einer aminoterminalen Deletion, wobei die Polypeptidsequenz bei einer beliebigen Aminosäure beginnt, die zwischen der LF1-Spleißstelle und der ersten Aminosäure des UCR1-Startpunktes der nativen langen Form des PDE4-Polypeptids liegt, wobei das Polypeptid erniedrigte Aggregatbildung aufweist.
2. Polypeptid nach Anspruch 1, wobei die PDE4-Polypeptidsequenz eine PDE4D-Polypeptidsequenz ist.
- 10 3. Polypeptid nach einem der Ansprüche 1 bis 2, wobei die Polypeptidsequenz an der LF1-Spleißstelle des nativen PDE4-Polypeptids beginnt.
4. Polypeptid nach einem der Ansprüche 1 bis 3, wobei sich der Startpunkt der PDE4-Polypeptidsequenz 13 Aminosäuren stromaufwärts des UCR1-Startpunktes des nativen PDE4-Polypeptids befindet.
- 15 5. Polypeptid nach einem der Ansprüche 2 bis 4, wobei die PDE4D-Polypeptidsequenz die Sequenz einer Isoform ist, ausgewählt aus der Gruppe bestehend aus D3, D4, D5, D6, D7 und D8.
- 20 6. Polypeptid nach den Ansprüchen 1 bis 5, wobei das Polypeptid eine oder mehrere Mutationen von Serinresten umfasst.
7. Polypeptid nach Anspruch 6, wobei die Serinreste entweder zu Alanin oder Asparaginsäure mutiert sind.
- 25 8. Polypeptid nach den Ansprüchen 6 oder 7, wobei die Serinreste ausgewählt sind aus der Gruppe bestehend aus Serinresten, die Ser 54 und Ser 579 in Seq ID NO. 1 entsprechen.
9. Polypeptid nach einem der Ansprüche 1 bis 5, wobei das Polypeptid erniedrigte Tubulinassoziation aufweist.
- 30 10. Polynucleotidsequenz, die ein Polypeptid nach den Ansprüchen 1 bis 9 codiert.
11. Expressionsvektor oder Virus, umfassend eine Polynucleotidsequenz nach Anspruch 10 und fähig, die Expression der Polynucleotidsequenz in einer kompatiblen prokaryontischen oder eukaryontischen Wirtszelle zu steuern.
- 35 12. Virus nach Anspruch 11, wobei das Virus ein rekombinantes Baculovirus ist.
13. Prokaryontische oder eukaryontische Wirtszelle, die mit dem Expressionsvektor oder Virus nach Anspruch 11 oder 12 transformiert oder infiziert ist.
- 40 14. Wirtszelle nach Anspruch 13, wobei die Wirtszelle eine Insektenzelle ist.
15. Verfahren für die Produktion eines Polypeptids nach den Ansprüchen 1 bis 9, umfassend die Züchtung der Wirtszelle nach den Ansprüchen 13 oder 14 in einem geeigneten Medium, so dass das Polypeptid exprimiert wird, und die Reinigung des Polypeptids aus den Zellen.
- 45 16. Verfahren für die Produktion eines Polypeptids nach Anspruch 15, wobei die Wirtszelle eine Insektenzelle ist.
17. Polypeptid nach den Ansprüchen 1 bis 9, das durch das Verfahren nach den Ansprüchen 15 oder 16 produziert wurde.
- 50 18. Screening-Assay zur Identifizierung von Agonisten oder Antagonisten von Phosphodiesteraseaktivität, umfassend mindestens eines der Polypeptide nach den Ansprüchen 1 bis 9.
19. Verfahren zur Identifizierung eines Wirkstoffkandidaten zur Therapie einer entzündlichen Erkrankung, umfassend die Messung der Aktivierung oder Hemmung der Phosphodiesteraseaktivität eines der Polypeptide nach einem der Ansprüchen 1 bis 9.
- 55 20. Verwendung eines Polypeptids nach einem der Ansprüchen 1 bis 9 zur Identifizierung eines Agonisten oder Antagonisten von Phosphodiesteraseaktivität.

21. Verwendung eines Polypeptids nach den Ansprüchen 1 bis 9 zur Kristallisation.

22. Kit zur Identifizierung von Agonisten oder Antagonisten von Phosphodiesteraseaktivität, umfassend mindestens eines der Polypeptide nach den Ansprüchen 1 bis 9 und einen radioaktiven oder IMAP-FP-Phosphodiesterase-Kit.

5

Revendications

- 10 1. Polypeptide comprenant une séquence polypeptidique d'une PDE4 sous forme longue avec une délétion amino-terminale, où la séquence polypeptidique commence à un aminoacide quelconque situé entre le site d'épissage LF1 et le premier aminoacide constituant le début du domaine UCR1 du polypeptide PDE4 sous forme longue natif, ledit polypeptide présentant une quantité d'agrégats diminuée.
- 15 2. Polypeptide selon la revendication 1 ladite séquence polypeptidique de PDE4 étant une séquence polypeptidique de PDE4D.
3. Polypeptide selon l'une quelconque des revendications 1 à 2, la séquence polypeptidique commençant au site d'épissage LF1 du polypeptide PDE4 natif.
- 20 4. Polypeptide selon l'une quelconque des revendications 1 à 3, le début de la séquence polypeptidique de PDE4 étant localisé 13 aminoacides en amont du début du domaine UCR1 du polypeptide PDE4 natif.
- 25 5. Polypeptide selon l'une quelconque des revendications 2 à 4, la séquence polypeptidique de PDE4D étant une séquence d'une isoforme choisie dans le groupe constitué par D3, D4, D5, D6, D7 et D8.
- 30 6. Polypeptide selon les revendications 1 à 5, ledit polypeptide comprenant une ou plusieurs mutations de résidus de sérine.
7. Polypeptide selon la revendication 6, dans lequel lesdits résidus de sérine sont mutés en alanine ou en acide aspartique.
8. Polypeptide selon les revendications 6 ou 7, dans lequel lesdits résidus de sérine sont choisis dans le groupe constitué par les résidus de sérine correspondant à Ser54 et Ser579 dans SEQ ID n° : 1.
- 35 9. Polypeptide selon l'une quelconque des revendications 1 à 5, ledit polypeptide présentant une association à la tubuline diminuée.
10. Séquence de polynucléotide codant pour un polypeptide selon les revendications 1 à 9.
- 40 11. Vecteur d'expression ou virus comprenant une séquence de polynucléotide selon la revendication 10 et capable de diriger l'expression de la séquence de polynucléotide dans une cellule hôte procaryote ou eucaryote compatible.
12. Virus selon la revendication 11, le virus étant un baculovirus recombiné.
- 45 13. Cellule hôte procaryote ou eucaryote transformée ou infectée par le vecteur d'expression ou le virus des revendications 11 ou 12.
14. Cellule hôte selon la revendication 13, la cellule hôte étant une cellule d'insecte.
- 50 15. Procédé pour la production d'un polypeptide selon les revendications 1 à 9 comprenant la culture de la cellule hôte des revendications 13 ou 14 dans un milieu approprié de telle sorte que ledit polypeptide soit exprimé, et la purification dudit polypeptide à partir des cellules.
- 55 16. Procédé pour la production d'un polypeptide selon la revendication 15, dans lequel la cellule hôte est une cellule d'insecte.
17. Polypeptide selon les revendications 1 à 9, produit par le procédé selon les revendications 15 ou 16.

- 18.** Test de criblage pour l'identification d'agonistes ou d'antagonistes de l'activité phosphodiesterase comprenant au moins un des polypeptides selon les revendications 1 à 9.
- 19.** Procédé pour l'identification d'un médicament candidat pour la thérapie d'une maladie inflammatoire, ledit procédé comprenant la mesure de l'activation ou de l'inhibition de l'activité phosphodiesterase d'un quelconque des peptides selon les revendications 1 à 9.
- 20.** Utilisation d'un polypeptide selon les revendications 1 à 9, pour identifier un agoniste ou un antagoniste de l'activité phosphodiesterase.
- 21.** Utilisation d'un polypeptide selon les revendications 1 à 9 pour la cristallisation.
- 22.** Trousse pour l'identification d'agonistes ou d'antagonistes de l'activité phosphodiesterase comprenant au moins un des polypeptides selon les revendications 1 à 9, et un kit de test de phosphodiesterase radioactif ou IMPP-FP.

Figure 1

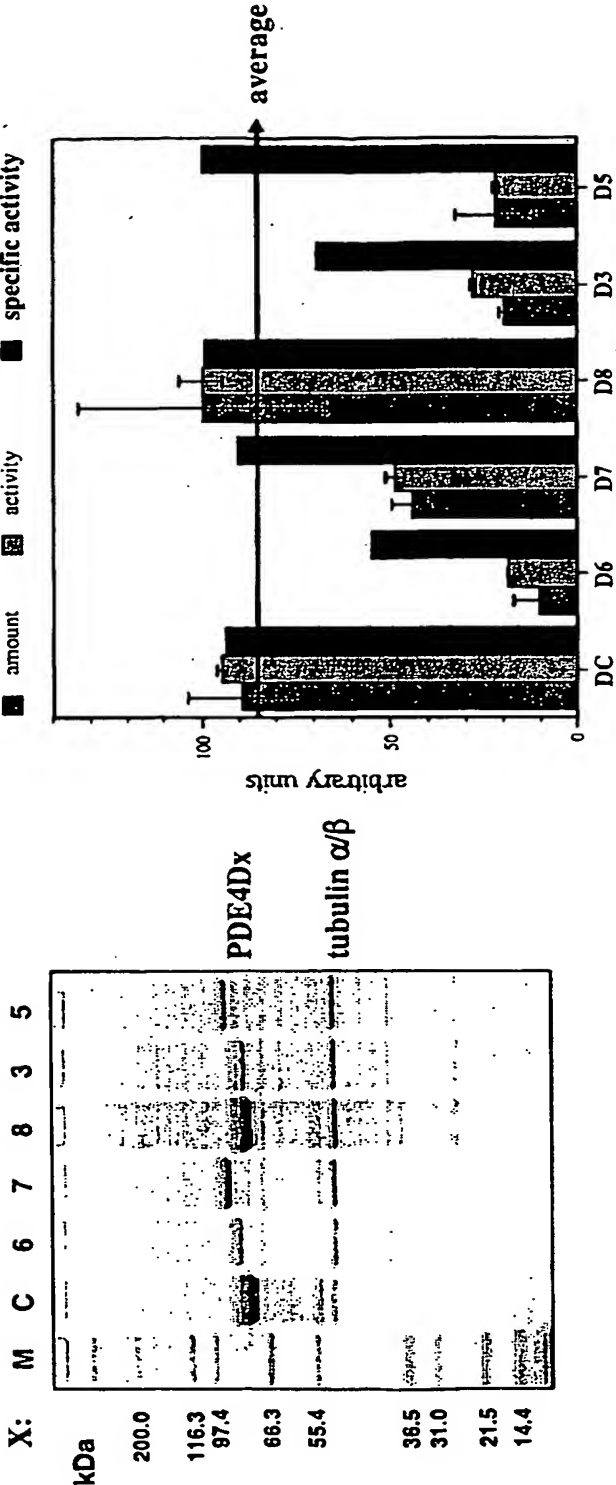


Figure 2

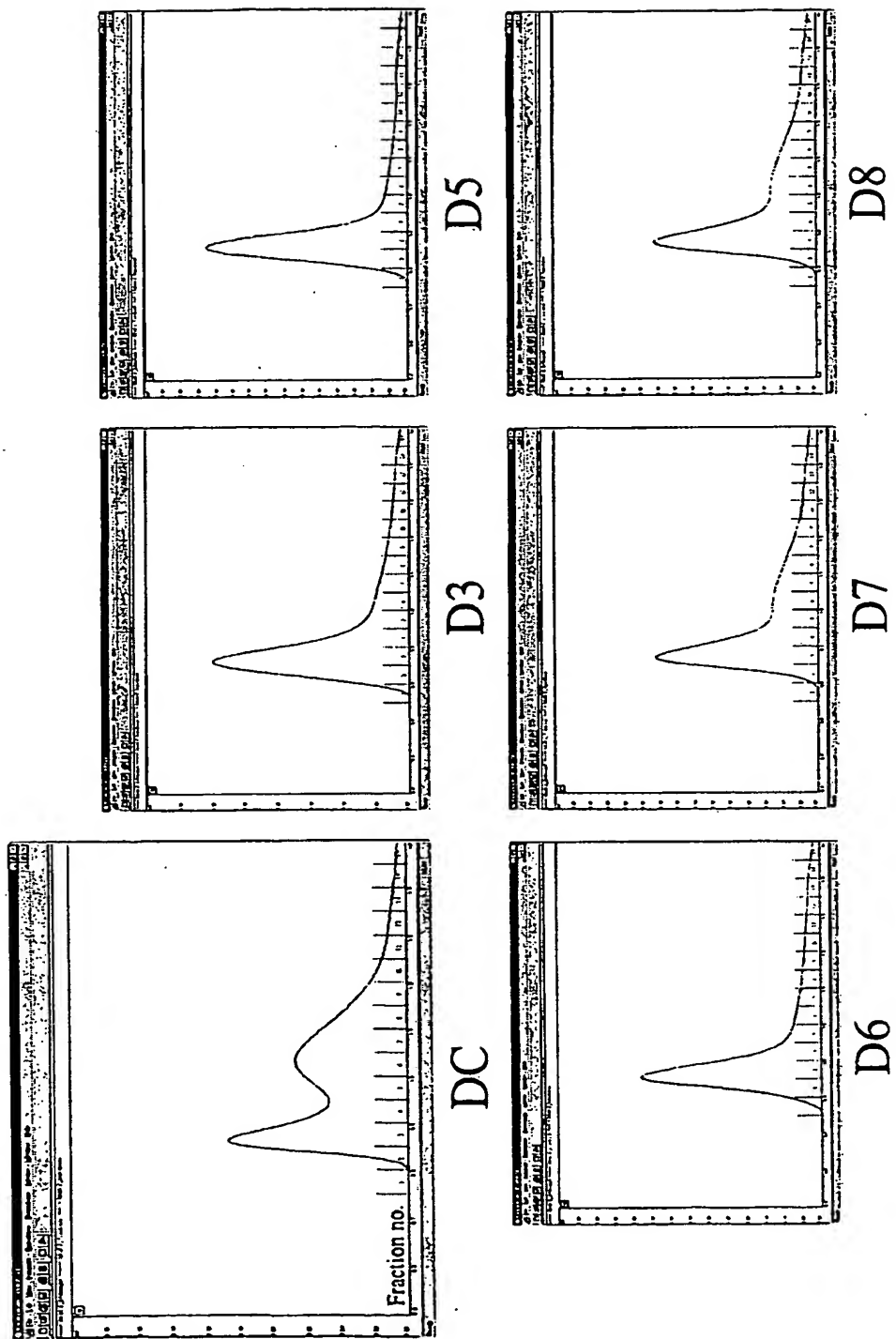


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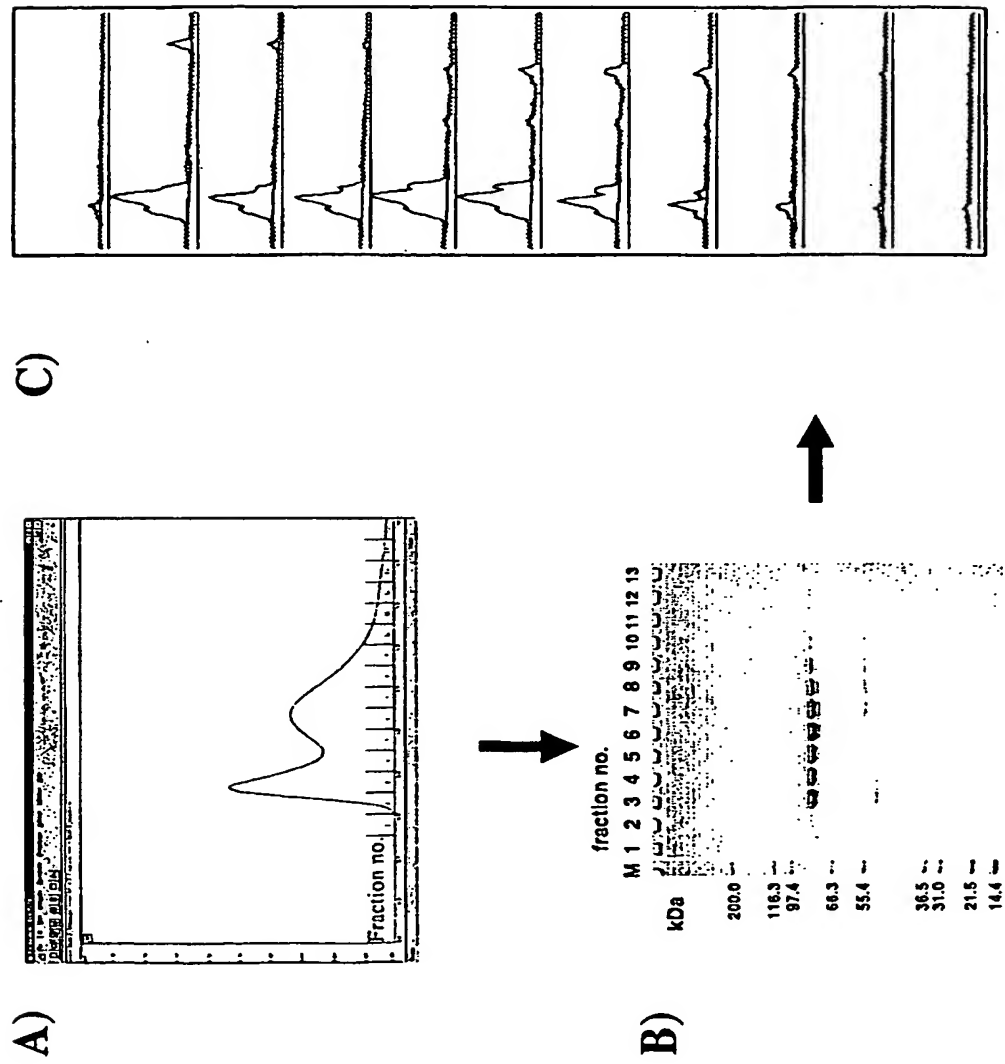
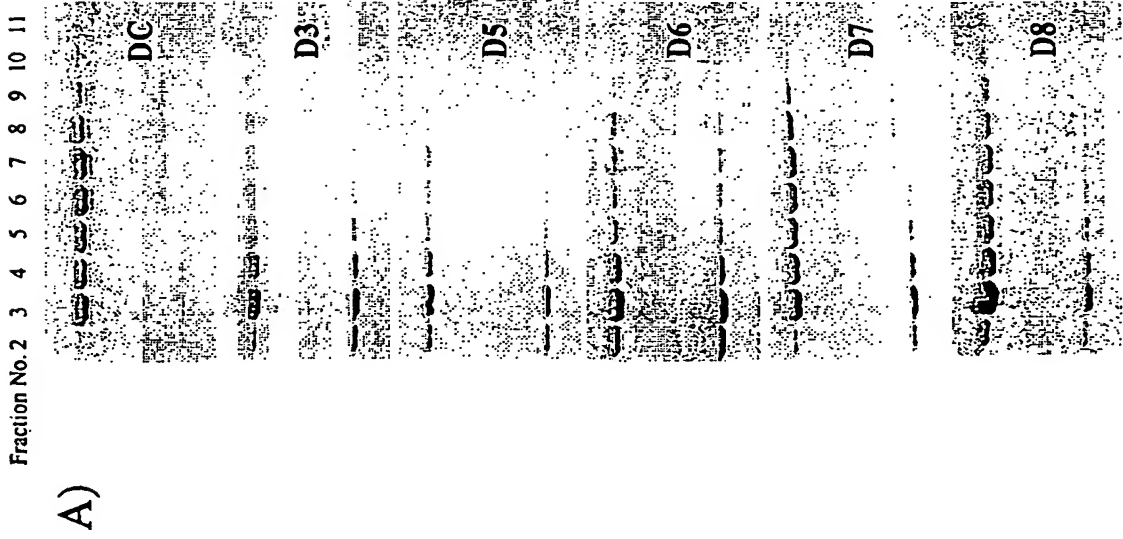
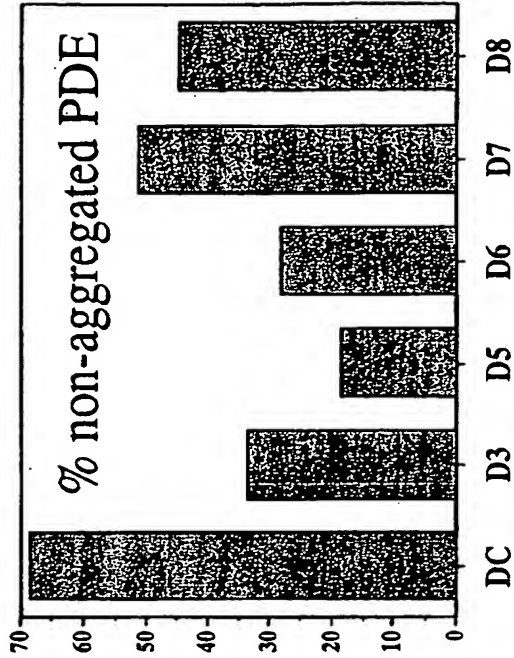


Figure 4



B)



C)

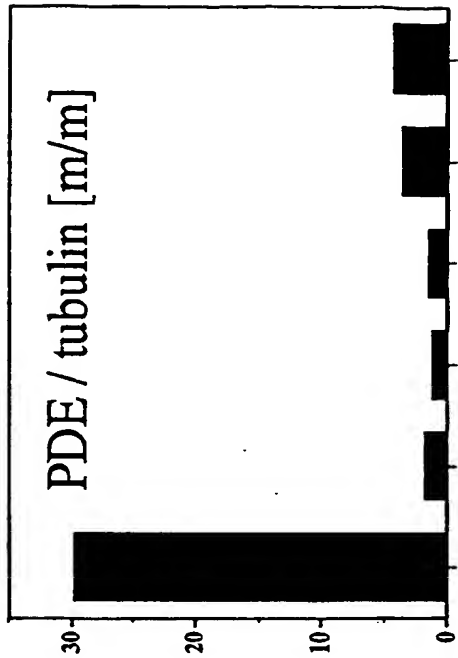


Figure 5

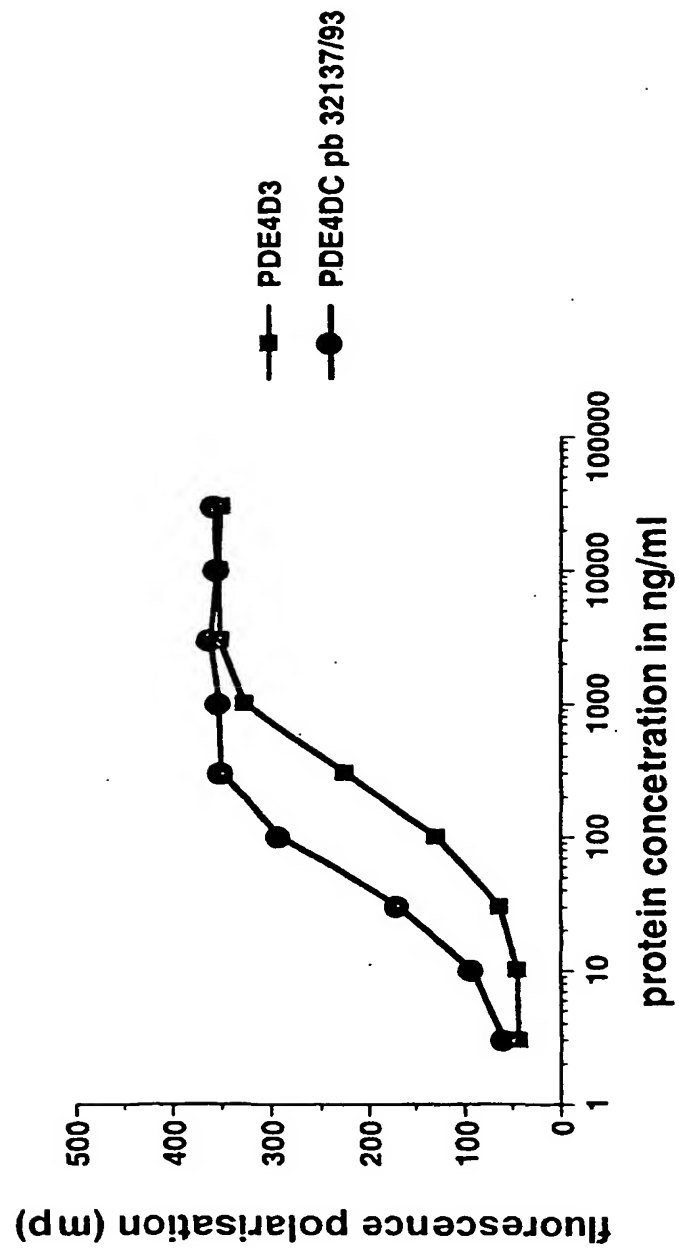


Figure 6
fluorescence polarization assay
30ng/ml PDE4D core construct IC₅₀ Rolipram @40nMcAMP

